EXHIBIT 4

U.S. Serial No. 10/750,005

Larsson, A. and Hagnefeldt, L., 1983, *Functions of Glutathione: Biochemical, Physiological, Toxicological, and Clinical Aspects*, Raven Press, NY, pp. 317-24

Functions of Glutathione: Biochemical, Physiological, Toxicological, and Clinical Aspects, edited by A. Larsson et al. Raven Press, New York © 1983.

Hereditary Glutathione Synthetase Deficiency in Man

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Glutathione has been postulated to be involved in a number of fundamental biological processes (1). If these postulates are correct, it is surprising that inborn errors of the metabolism of glutathione are encountered in clinical medicine. Patients with such disorders do, however, exist and the field has recently been reviewed (9,13). Clinical and biochemical studies of these patients are necessary in order to provide optimal therapy. In return, studies of the patients can give important information about the role of glutathione in different biological processes.

Human genetic defects have been identified in four of the six enzymes of the γ-glutamyl cycle (Fig. 1) (9,13) affecting the following enzymes: γ-glutamylcysteine synthetase, glutathione synthetase, γ-glutamyltranspeptidase, and, recently, 5-oxoprolinase (A. Larsson et al. *this volume*; 12,17). In this chapter, we will focus on one inborn error of glutathione metabolism, namely human glutathione synthetase deficiency. The same disorder is discussed by E. Jellum et al., *this volume.*

HETEROGENEITY OF GLUTATHIONE SYNTHETASE DEFICIENCY

The first patients with hereditary glutathione synthetase deficiency were reported by Oort et al. (14) and Prins et al. (16). The only symptom of these patients was compensated hemolytic anemia, and their erythrocytes contained decreased levels of glutathione. Additional patients with this inborn error have subsequently been identified, and it should be emphasized that they have no neurological symptoms, no metabolic acidosis, and no 5-oxoprolinuria (2). This is in contrast to another group of patients with glutathione synthetase deficiency discussed below.

The biochemical heterogeneity of hereditary glutathione synthetase deficiency was studied by Spielberg et al. (20). These authors found that in the oxoprolinuric form erythrocytes, as well as leukocytes and cultured fibroblasts,

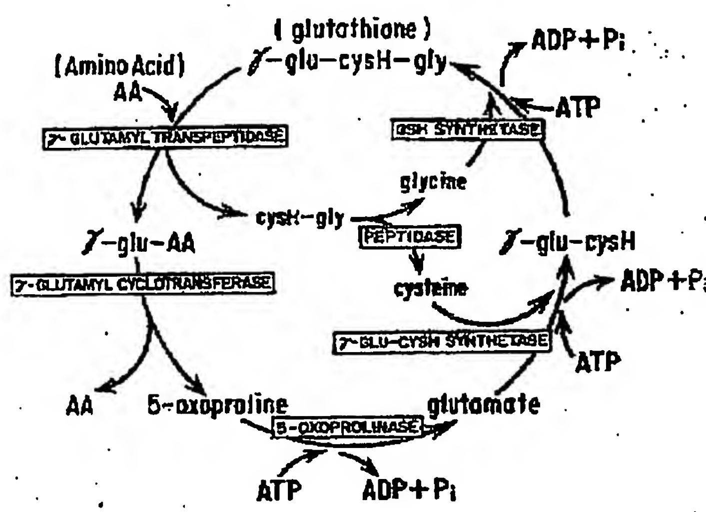


FIG. 1. γ-Glutamyl cycle from Wellner et al. (21).

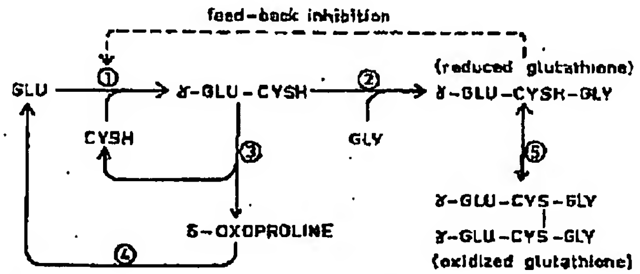
contained markedly decreased enzyme levels. In the nonoxoprolinuric form the enzyme defect was expressed only in erythrocytes. It was speculated that the latter group of patients carry a mutation, which mainly affects the stability of the enzyme, whereas in the oxoprolinuric form the mutation affects the catalytic activity.

GENERALIZED GLUTATHIONE SYNTHETASE DEFICIENCY

Clinical Signs and Symptoms

Generalized glutathione synthetase deficiency has so far been reported in 12 patients (9). The disease has autosomal recessive inheritance.

The patients are usually detected in the neonatal period, and their main symptoms are metabolic acidosis and jaundice. Acidosis correction is usually required and life-long substitution by daily oral doses of sodium bicarbonate or citrate is often necessary. The neonatal jaundice usually reflects an increased rate of hemolysis, and exchange blood transfusions are sometimes required because of neonatal hyperbilirubinemia.



iG. 2. Mechanism of 5-oxoproline overproduction in patients with glutathione nthetase deficiency. Enzymatic steps involved in the biosynthesis of glutathione:) γ-glutamylcysteine synthetase; (2) glutathione synthetase; (3) γ-glutamylcyclo-ansferase; (4) 5-oxoprolinase; (5) glutathione reductase. From Larsson and attsson (10), with permission of Elsevier/North Holland.

The excretion of 5-oxoproline (pyroglutamic acid) in the urine is usually cessive. This is caused by an overproduction of 5-oxoproline (8) due to lack feedback inhibition of reduced glutathione in γ-glutamylcysteine synthetase, d subsequent degradation of the γ-glutamylcysteine to 5-oxoproline by glutamylcyclotransferase (10,21) (Fig. 2). In some patients the 5-oxoproline cretion is in the order of 200 mmoles (26 g) per day 1.73 m^2 body surface a, while other patients excrete only one-tenth of that amount. This variation 5-oxoproline excretion most likely reflects differences in the degree of ficiency of glutathione synthetase in critical tissues. The acidosis is nsidered to reflect the accumulation of 5-oxoproline in body fluids; blood els of 5-oxoproline have been reported to be in the range of 1 to 5 mmoles/ r.

Postnatally, the dominating symptoms are a chronic metabolic acidosis and a w but progressive damage of the central nervous system. The patients scribed have been observed at different ages, ranging from 6 months to 28 rs, and their intellectual development and neurological symptoms have ied. However, 8 of 12 patients have shown mental retardation and 7 of the ients had additional neurological symptoms (9).

The oldest patient was a man who died at 28 years of age with multiple signs central nervous system damage such as ataxia, seizures, spasticity, and ious mental retardation. At autopsy selective atrophy of the granule cell layer the cerebellum was found, as well as focal cortical lesions (E. Jellum et al., s *volume*, 18).

We have recently observed a progressive decline in the intellectual develop-nt of two Swedish sisters aged 11 and 8 years. Their developmental test

results have gradually decreased from quotients of 100 to 115 to levels of 80 to 90. Furthermore, both girls exhibited pathological electroretinograms with decreased oscillatory potentials and low A and B wave amplitudes. Details of these observations will be reported elsewhere.

Two infants have died in therapy resisting attacks of acidosis in association with infections (2,15).

An interesting finding in one patient with generalized glutathione synthetase deficiency was reported by Boxer et al. (3). Their patient, a two-year-old boy, had recurrent episodes of bacterial infections, which were most likely due to abnormal granulocyte function. The leukocyte function was normalized after treatment with high doses of vitamin E.

Accumulation of γ-Glutamylcysteine

It seemed likely that some γ-glutamylcysteine could accumulate intracellularly in patients with glutathione synthetase deficiency. This dipeptide contains both functional groups—the sulfhydryl and the γ-glutamyl—of glutathione. It was earlier speculated that γ-glutamylcysteine might in fact substitute for glutathione in different processes (21).

We tested the possibility that γ-glutamylcysteine accumulated in glutathione synthetase deficiency by studying erythrocytes (7). Erythrocytes from the deficient patients had γ-glutamylcysteine levels within the range for control cells (66 ± 24 μmol/l; mean $\pm$ SD). Thus, the activity of γ-glutamylcyclotransferase in the mutant cells seemed to be high enough to clear the intracellular compartment of γ-glutamylcysteine by conversion to 5-oxoproline and cysteine.

In subsequent studies of fibroblasts, however, cells from patients with glutathione synthetase deficiency were found to contain more low molecular weight thiol compounds than was accounted for by glutathione (5). In control fibroblasts, on the other hand, there was good agreement between nonprotein SH and glutathione.

We have now analyzed the concentrations of low molecular weight thiols (and disulfides) in extracts of cultured fibroblasts using high performance liquid chromatography (11) (Table 1). Significant accumulation of γ-glutamylcysteine was found in cells from the patients; in these cells 70 to 90% of the low molecular weight sulfhydryl compounds were accounted for by γ-glutamylcysteine. In control cells the level of γ-glutamylcysteine was about 20% of the total low molecular weight sulfhydryl residues.

Cultured fibroblasts were also labeled with ^{35}S-cysteine for up to 3 hr (11). *N*-Ethylmaleimide (NEM) was then added and the proteins were denatured by trichloroacetic acid. The extracts were subjected to high-voltage paper electrophoresis (Fig. 3). In control cells two main peaks were found corresponding to the NEM derivatives of glutathione and cysteine; occasionally a small peak was found corresponding to NEM-γ-glutamylcysteine. In cells from

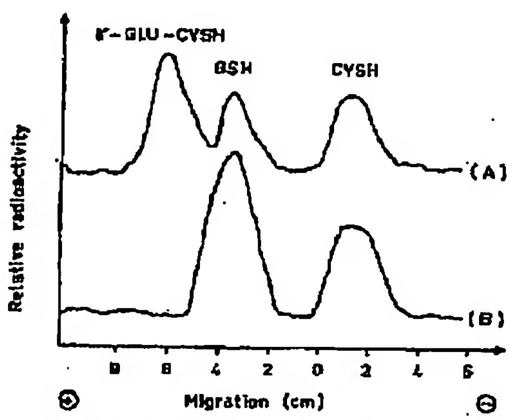
TABLE 1. Nonprotein SH (NPSH), glutathione (GSH), and γ-glutamylcysteine (γ-GLU-CYSH) in cultured fibroblasts from control subjects and patients with glutathione synthetase deficiency

Origin of cells	NPSH[a]	GSH[a]	γ-GLU-CYSH[a]
Control subjects ($N = 4$)	15.2 ± 5.1	9.0 ± 1.2	2.8 ± 0.1
Patients ($N = 3$)	8.4 ± 2.1	2.1 ± 0.8	6.7 ± 0.8

[a]Protein = nmole/mg. From Larsson et al. (11).

patients we consistently found a pattern of three peaks as shown in Fig. 3. The major fraction of the ^{35}S-activity was found in γ-glutamylcysteine, especially early during the incubation.

On the basis of these findings we would like to postulate that γ-glutamylcysteine might accumulate in tissues other than erythrocytes in patients with generalized glutathione synthetase deficiency. γ-Glutamylcysteine might in fact substitute for glutathione, especially in tissues with low activity of γ-glutamylcyclotransferase, thereby protecting the patients from oxidative damage. Unfortunately, brain appears to have higher γ-glutamylcyclotransferase activity than any other tissue studied (4), which may have relevance for the development of central nervous system damage in patients with generalized glutathione synthetase deficiency.



3. S. High-voltage paper electrophoresis of ^{35}S-labelled N-ethylmaleimide-derivatives in the acid-soluble fraction of cultured fibroblasts from a patient with glutathione synthetase deficiency (A) and from a control subject (B). The cells were incubated with ^{35}S-cystine for 90 min (11).

Therapeutic Trial with Mercaptopropionyl-Glycine

The fact that our two patients progressively declined in intellectual development strongly indicates that they needed more active therapy than mere acidosis correction with bicarbonate.

Administration of massive doses of vitamin E to patients with glutathione synthetase deficiency apparently corrected the defective granulocyte function (3), and also increased erythrocyte survival (19). However, even when vitamin E treatment was started in the neonatal period (in one patient) it did not prevent the psychomotor retardation at 2 to 3 years of age (J. D. Schulman, *personal communication*).

As discussed previously, we were anxious to avoid any treatment that might inhibit the synthesis of γ-glutamylcysteine. Thus, administration of cysteamine was considered hazardous (6).

The possibility of using α-mercaptopropionylglycine (Thiola®, Santen Pharmaceutical Company, Osaka, Japan) was brought to our attention by Dr. L. Révész. Oral administration of this sulfhydryl compound is indicated in a variety of disorders, one of these being liver disease (22). Apparently the toxic side effects of α-mercaptopropionylglycine are considerably less than for instance penicillamine. Furthermore, α-mercaptopropionylglycine did not inhibit γ-glutamylcysteine synthetase in hemolysates. The patients received about 10 mg/kg/day, divided in 3 doses over a period of 4 months, and thereafter 20 mg/kg/day for one month. A number of clinical, biochemical, and neurophysiological parameters were monitored. In summary, we did not see any positive effects: Urinary excretion of 5-oxoproline did not change, and the electroretinograms remained pathological.

The trial with α-mercaptopropionylglycine has now been terminated and a new trial involving vitamin E administration has been started.

CONCLUSIONS

The clinical picture that emerges in generalized glutathione synthetase deficiency is chronic metabolic acidosis, 5-oxoproline overproduction, hemolytic anemia, which is often well compensated, and progressive central nervous system damage. The CNS involvement justifies therapeutic trials along several lines. Ideally, glutathione should be administered, but probably has to be given parenterally, and it is doubtful if it even then reaches the CNS. We have tried to give α-mercaptopropionylglycine orally, 10 to 20 mg/kg/day, over months without any positive effects in two patients.

Studies in cultured fibroblasts have shown that there are prerequisites for the accumulation of γ-glutamylcysteine in different tissues in patients with glutathione synthetase deficiency. Incidentally, this was not revealed by studies of erythrocytes. The accumulation of γ-glutamylcysteine may be beneficial, since the dipeptide can possibly substitute for glutathione in different processes.

Therapeutic interventions aiming at inhibition of γ-glutamylcysteine synthetase might therefore be hazardous.

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Medical Research Council (Projects No. 4792, 6943), and Svenska Livförsäkringsbolags Nämnd för Medicinsk Forskning.

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